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Abstract

This study describes the synthesis, characterization and *in vitro* evaluation of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-gadolinium (Gd) – doxorubicin (Dox) conjugates. Copolymers of HPMA were derivatized to incorporate side chains for Gd chelation and Dox conjugation. The conjugates were characterized by their side-chain contents, T_1 relaxivity (r1), stability, and *in vitro* cytotoxicity. High stability and relaxivity of these conjugates coupled with low toxicity show their potential for monitoring the *in vivo* fate of HPMA-based drug delivery systems by magnetic resonance imaging techniques.

Keywords: HPMA Copolymers, Contrast Agents, Magnetic Resonance Imaging, Relaxivity, Drug Delivery

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Introduction

Detection and prediction of the fate of drug delivery systems within the tumor is of critical importance in cancer therapy. Prediction of the fate of drug delivery systems derived from standard pharmacokinetic models is frequently inadequate because of the complex nature of tumor blood flow and microenvironment. Although tissue drug concentrations within the tumor and non-target organs can be sampled with microdialysis [1] or biopsy, noninvasive alternatives for evaluating the distribution of polymeric drug delivery systems are yet to be developed.

To date, the most commonly used noninvasive imaging modalities are nuclear,[2,3] magnetic resonance (MR),[4,5] and optical techniques.[6-8] Among these approaches, magnetic resonance imaging (MRI) combines the benefits of high spatial resolution [9] with unique capability to simultaneously elicit both anatomic and physiological information.

The use of magnetic resonance contrast agents to enhance the contrast of images in medical imaging is critical. Low molecular weight gadolinium complexes, are currently used as extracellular magnetic resonance contrast agents in a large fraction of clinical examinations. Several macromolecular contrast agents are in preclinical and clinical trials.[10] These are of interest because they have a prolonged blood pool retention time and can leak out only in compromised endothelium. Due to hyperpermeability of neoplastic blood vessels in tumor tissues,[11,12] macromolecular contrast agents show potential for imaging and characterization of tumors. Accumulation of macromolecules at the tumor site via "enhanced permeability and retention" (EPR) effect [12] allow targeting of anticancer drugs to solid tumors. Consequently, by attaching both the contrast agent

and the chemotherapeutic agent to the polymeric side chains, one can follow the fate of polymeric drug conjugates and subsequent correlation with treatment.

N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are non-toxic, non-immunogenic water soluble polymeric carriers that are in various stages of clinical trials for cancer therapy.[13] Previously the potential of HPMA copolymers for passive and active delivery of contrast agents was reported.[14-17] In this study we report the synthesis, characterization, *in vitro* stability and cytotoxicity of a macromolecular drug delivery system based on HPMA copolymer containing both a chemotherapeutic drug and an MRI contrast agent.

Chemicals and reagents

N, N'-azobisisobutyronitrile (AIBN) and gadolinium (III) chloride hexahydrate ($GdCl_3.6H_2O$) were obtained from Aldrich (Milwaukee, WI, USA). *N*-(3-aminopropylmethacrylamide (APMA) was obtained from Polysciences, Inc. (Warrington, PA, USA). p-isothiocyanatobenzyl-1, 4, 7, 10 tetraazacyclododecane-1, 4, 7, 10 tetraacetic acid (p-SCN-Bz-DOTA) was obtained from Macrocyclics (Dallas, TX, USA). *N, N, N', N'*- ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate was obtained from USB Corporation (Cleveland. OH, USA), fetal bovine serum from QBI (Gaithersburg, MD, USA) and Calf bovine serum from ATCC (Manassas, VA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) and were of reagent grade.

Cell culture

Human breast cancer cell line MDA-MB-435 (ATCC HTB-129; ATCC, Manassas, VA) was cultured in DMEM (ATCC 30-2002) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin. Mouse fibroblast cell line

NIH/3T3 (ATCC CRL-1658; ATCC, Manassas, VA) was cultured in DMEM supplemented with 10% heat inactivated calf bovine serum. Both cell lines were grown at 37 °C in a humidified atmosphere of 5% CO_2.

Synthesis and characterization of polymer-drug-contrast agent conjugates

Monomer synthesis

Methacryloylglycylphenylalanylleucylglycyl-doxorubicin (MA-GFLG-Dox)[18] and *N*-(2-hydroxypropyl)methacrylamide (HPMA),[19] were prepared as described previously. Comonomer aminopropylmethacrylamide-benzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (APMA-benzyl-DOTA) was synthesized by reacting *N*-(3-aminopropylmethacrylamide) (APMA) with p-isothiocyanatobenzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (p-SCN-Bz-DOTA) in dry dimethylsulfoxide (DMSO). The p-SCN-Bz-DOTA was reacted at 1.2 molar excess to APMA.

Polymer synthesis

HPMA copolymer conjugates with Dox (P-(DOTA-Gd)-Dox) and without Dox (P-(DOTA-Gd)) (Table 1) were synthesized by a modified two-step procedure. In the first step the polymer backbones were synthesized by free radical precipitation copolymerization of the monomers of HPMA, APMA-benzyl-DOTA, and MA-GFLG-Dox in predetermined molar compositions (Table 1). All polymerizations were carried out in acetone / DMSO using AIBN as the initiator. 3-mercaptopropionic acid (0.01 mole%) was used as a chain terminating agent to control the molecular weight of HPMA

copolymer-DOTA conjugate. The ratio of monomers: initiator: solvent in the feed were kept constant at 12.5: 0.6: 86.9 (weight %), respectively. The comonomer mixture was sealed in an ampoule under nitrogen and stirred at 50 °C for 24 h. The polymers were isolated by precipitation of resulting solution into ether. The contents of side chains terminating in Dox were determined by UV spectrophotometry (λ_{max} = 482 nm).

In the second step, gadolinium (Gd) was chelated to side chain of the polymers terminating in DOTA as described elsewhere.[15] Briefly HPMA copolymer-DOTA conjugates and $GdCl_3.6H_2O$ (1.5:1 molar equivalents relative to the DOTA content of the feed) were dissolved in aqueous solution at pH 5.0-5.5. EDTA disodium salt dehydrate (EDTA:Gd, 1:1) was added into the solutions to chelate the excess and non-specifically bound Gd. After stirring for 30 min, the solution was purified over a PD10 size exclusion column (GE Healthcare, NJ, USA), to remove the EDTA-chelated Gd and other unreacted low molecular weight monomers from the polymeric conjugates. The polymer conjugates were dissolved in deionized water, dialyzed and lyophilized. The chemical structure of a typical polymeric construct is shown in Figure 1.

Physicochemical characterization

Polymer-contrast agent complexes were characterized for their Gd content by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Galbraith, Knoxville, TN). Dox content of the P-(DOTA-Gd)-Dox conjugate was determined by UV spectrophotometry at 482 nm. The molecular weight and molecular weight distribution of the polymeric conjugates were estimated by size exclusion chromatography (SEC) on a Superose 12 HR 10/30 column (GE Healthcare, Piscataway, NJ) using a Fast Protein

Liquid Chromatography (FPLC) system (GE Healthcare) and HPMA homopolymer fractions of known molecular weight as calibration standards.

Relaxivity measurements

The r_1 relaxivity of HPMA copolymer-(DOTA-Gd) chelates were calculated from T_1 (relaxation time) measurements at room temperature. Solutions of each sample were diluted in deionized water at four concentrations (from 0.1 to 0.015 mM) and were imaged using 1.5 T MRsystem (Eclipse, Philips Medical System, Cleveland, OH). T_1 was measured by an inversion recovery fast spin echo imaging sequence using inversion times (TI) of 50, 100, 200, 400, 700, 1400, 2000, and 2800 ms, an echo time (TE) of 12 ms, and an echo train length of 8 at a repeat time TR of 6000 ms. All images were obtained from a single axial slice with a 20×15 cm field of view (FOV), 3 mm slice thickness, 256×192 matrix and one excitation. Images were transferred to an independent workstation (SGI, O200) for the calculation of T_1 from the images obtained at various inversion times. T_1 for each solution and deionized water were calculated using MATLAB (The Mathworks, Inc., Natick, MA). The r_1 values of each solution were calculated, using a least squares fit, as the slope of $(1/T_{1,\,solution} - 1/T_{1,\,water})$ versus concentration of contrast agent (mM), where $T_{1,\,solution}$ is the T_1 of each dilution of the contrast agent and $T_{1,\,water}$ is the T_1 of water without contrast agent.

Stability of P-(DOTA-Gd) complex

The stability of the HPMA copolymer- contrast agent complex was evaluated across a range of pH (Table 2). Aliquots of 2mg/ml HPMA- contrast agent complex were

incubated at pH 3, 5 and 7 for 1, 3 and 5 days at room temperature. At each time point samples were eluted in a PD10 size exclusion column (GE Healthcare, NJ, USA), to remove decomplexed Gd and other low molecular weight impurities. The samples were lyophilized and Gd contents of polymeric conjugates were measured by ICP-OES. Results were reported as % Gd bound compared to (P-(DOTA-Gd) (Table 1). The free Gd content of the polymers was determined using Arsenaso III assay.[20]

The stability of Gd-DOTA complex was also evaluated in presence of a range of excess concentrations of a competitive chelator namely EDTA (Table 3). Briefly polymer-DOTA conjugates and $GdCl_3.6H_2O$ (1.5:1 molar equivalents relative to the DOTA content of the feed) were dissolved in deionized water. The pH of the solution was maintained at 5.0-5.5 overnight by gradual addition of 1 N NaOH solution. The solution was divided into four equal volumes and EDTA disodium salt dihydrate was added at 1, 5, 25, and 125 times of Gd concentration. Polymeric solution with (1:1 EDTA:Gd) concentration was treated as a control. After stirring for 30 min, the solutions were eluted in a PD10 size exclusion column (GE Healthcare, NJ, USA) to remove the EDTA-chelated Gd and other unreacted low molecular weight monomers from the polymeric conjugate. The samples were lyophilized and Gd contents of polymeric complexes were measured by ICP-OES. Results were reported as % Gd bound compared to (P-(DOTA-Gd) (Table 1). The free Gd content of the polymers was determined using Arsenaso III assay.[20]

Cytotoxicity of polymeric complexes

The toxicity of polymeric conjugates was assessed using model breast cancer (MDA-MB-435) and non-cancerous fibroblast (NIH/3T3) cell lines. Cells were seeded on

96-well culture plates at a concentration of 3000 cells/ well and allowed to attach for 24 h at 37 °C and in humidified atmosphere of 5% CO_2. Subsequently, the medium was removed and 100 μl of HPMA copolymer-(DOTA-Gd) conjugate in DMEM (10% serum) was added to obtain final concentrations of 1 to 1000 μM Gd equivalent. MTT assay was performed at 24, 48, and 72 h to determine time dependent effects on toxicity. The same experiment was performed with HPMA copolymer-DOTA-Dox conjugates (with and without Gd) at concentrations between 1 to 10,000 nM Dox equivalent. Cells were assayed at 560 nm on a microplate reader (SPECTRAmax plus, Molecular Devices, Sunnyvale, CA). The toxicity of the conjugates in all experiments was expressed as % of viable cells. Statistical significance of differences in toxicity between different samples was analyzed using two-tailed unpaired student t-test.

Results

Characterization of conjugates

Characteristics of HPMA-(DOTA-Gd) complexes with and without doxorubicin are summarized in Table 1. The content of MA-GFLG-Dox in the copolymers was 0.26 mmol/g corresponding to 92% of the feed comonomer content. Subsequent chelation of Gd to the DOTA side chains of the conjugates resulted in Gd incorporation efficiency of 51 and 87% of the DOTA molecules per polymer backbone with and without Dox respectively. Both HPMA-linked Gd conjugates exhibited relaxation (r_1) values higher than the commercially available Gd-DOTA contrast agent (Dotarem®).[21] Polymeric -Gd complex with Dox exhibited 1.6 times higher relaxivity than polymeric complex without Dox. The estimated weight average molecular weight of the polymers was 35 and 34 kDa

with polydispersity index of 1.6 and 1.4 (Table 1), typical of similar polymeric conjugates reported in the literature.[14-16]

Chelate stability as a function of pH

Stability studies were performed in physiological and acidic pH conditions. Results showed that at pH 7 and 5 less than 5% of Gd was decomplexed in 5 days suggesting the high kinetic stability of the HPMA copolymer (DOTA-Gd) complex. At these pH values decomplexation was not observed beyond 3 days. At pH=3, 85.8% of Gd remained chelated after 5 days (Table 2). Arsenazo III assay showed less than 2% free Gd in each sample suggesting more than 98% of Gd was chelated in each final sample.

Competitive chelate challenge study

Gd labeling in the presence of increasing concentrations of a competitive chelator EDTA, resulted in lower Gd content of polymeric complexes (Table 3). The amount of Gd decomplexed over 30 min increased linearly with respect to the concentration of added EDTA compared to control. Arsenazo III assay showed less than 2% free Gd in each sample after 30 min incubation with EDTA and purification, suggesting more than 98% of Gd was chelated in final samples.

Cytotoxicity of polymeric complexes

The results of time- and concentration- dependent cytotoxicity of HPMA-(DOTA-Gd) complex (without Dox) on MDA-MB-435 is presented in Figure 2. Toxicity was represented as percentage of viable cells following treatment with the polymeric system and was compared to Magnevist, a commercially available Gd chelate contrast agent, at

incremental polymer concentrations (1, 10, 100 and 1000 μM Gd equivalent). At concentrations between 1 and 100 μM, polymer–Gd chelate showed significantly lower toxicity compared to Magnevist (Gd-DTPA) after 72 h ($p<0.019$). No significant differences between these compounds after 24 and 48 h at concentrations between 1 and 100 μM equivalent of Gd were observed. At 1000 μM Gd equivalent concentration polymer-Gd complex showed significantly higher toxicity than Magnevist after 48 and 72 h ($p<0.025$). No significant difference between these compounds was observed after 24 h at this concentration. The same experiment was performed on NIH/3T3 cell line. Although after 72 h, polymer-Gd complex showed a higher trend in % of viable cells on healthy fibroblast cell line, at each concentration there was no significant difference between the cytotoxicity of the same conjugates (Figure 2).

Toxicity of HPMA-DOTA-Gd conjugates with and without Dox on MDA-MB-435 cell line was compared to each other at 24, 48 and 72 h (Figure 3). No significant difference in toxicity of polymer-drug conjugates with and without Gd was observed, suggesting Gd does not interfere with the effect of Dox. Toxicity of polymeric Dox conjugate is significantly less than free Dox suggesting a slower endocytic mechanism of uptake for the conjugates compared to rapid diffusion of free Dox.

Discussion

The use of polymeric conjugates to selectively deliver cytotoxic anticancer drugs to tumor tissues is well established.[22] Water soluble polymer anticancer drug conjugates have demonstrated good aqueous solubility, increased half-life in the body, high antitumor effects and lower toxicity. These systems have the advantages of passive as well as active targeting of the tumor tissues.[23] Several HPMA copolymer based anti-

cancer agents are currently in Phase I/II clinical trials.[22] Non-invasive imaging methods of quantifying *in vivo* pharmacokinetics of these copolymers were developed using scintigraphic imaging of γ-emitting isotopes and SPECT.[24-30] Despite the use of radiolabled HPMA copolymers a number of shortcomings of isotopes limit their clinical utility.[31] These limitations include radiation, low tissue penetration, and high cost. Therefore, non-invasive methods for imaging of such drug delivery systems for correlation of localization with therapy needs to be developed.

MRI is a powerful noninvasive diagnostic modality that can provide high quality anatomic images and physiological data. The most significant advantage of MRI compared to scintigraphy is its high spatial resolution. Correlation of a detailed map of the delivery system deposition within the tissue with the local pathologic features, may help optimize the structure of the polymeric drug conjugates for personalized medicine. Therefore, components of the delivery system, such as molecular weight, charge, targeting moiety, drug content, drug releasing mechanism etc, may be adjusted to achieve superior therapeutic effect in individual patients. Although MRI is less quantitative than some other imaging modalities such as PET (positron emission tomography) and CT (computed tomography), the limitation concerning ionizing radiation does not apply to MRI.

Advances in applications of MRI for cancer imaging have depended predominantly on the use of contrast agents to enhance the appearance of the lesions. The most widely used contrast agents are chelates of gadolinium that have a strong magnetic field. However, the pharmacokinetic properties of these low molecular weight agents limit their application in many cases including cancer imaging. Attachment of Gd to

macromolecular carriers improves blood retention time and accumulation at tumor site because of hyperpermeability of neoplastic blood vessels. [11,12] Macromolecular contrast agents have potential for improved blood pool pharmacokinetics and MR contrast enhancement when compared to low molecular weight contrast agents.[32,33]

In this study, we evaluated a multifunctional macromolecular delivery system based on water-soluble HPMA copolymers, consisting of a contrast agent gadolinium (Gd) and a chemotherapeutic agent doxorubicin (Dox). Dox was conjugated to the polymeric backbone via a lysosomally degradable peptide spacer (GFLG). The idea is that by attaching both a chemotherapeutic and an imaging agent to the same polymeric carrier it is possible to develop compounds with a relaxivity suitable for MR imaging of the fate of the drug delivery system at the tumor site. This will allow us to correlate the time and extent of tumor localization on one hand and efficacy and toxicity on the other. The insight gained with this information can further be used to evaluate the effectiveness of therapy in individual patients based on tumor conditions (size, vascularization) and cancer stage.

HPMA copolymers are advantageous as macromolecular carriers because of the ability to tailor make the polymer backbone and control the content of side chains by facile chemical manipulations.[13] Previously it was shown that Gd chelated to HPMA copolymers can be used to monitor diseases such as rheumatoid arthritis and breast cancer.[15,17] The long circulation and local accumulation of these contrast agents allowed the performance of more detailed imaging procedures. Here, we designed a polymeric contrast agent containing doxorubicin as a model chemotherapy agent to evaluate the

relaxivity, stability, cytotoxicity and physicochemical properties of drug containing HPMA copolymer-Gd complex.

The average molecular size of the conjugates (~34.5 kDa) was lower than the glomerular filtration threshold of 45 kDa for HPMA copolymers.[34] This size is considered optimal for effective clearance of the polymer from the body over time. Observed relaxivities for HPMA copolymer contrast agent conjugates were improved over commercially available contrast agent Gd-DOTA (Table 1). Conjugation of Gd-DOTA to larger macromolecules is known to increase relaxivity by reducing rotational correlation time.[35] This has been observed for many Gd-based complexes[36-40] as well as HPMA based contrast agents.

Relaxivity of HPMA copolymer (DOTA-Gd)-Dox conjugate (Table 1) was higher than HPMA copolymer-Gd conjugate probably due to hydrophobic interactions between Dox molecules that may lead to inter- and intra-molecular interactions with overall slower local motions and global rotation. Hence, it may be possible to monitor the sustained incremental accumulation of HPMA copolymer-Dox conjugates at the tumor site with relatively higher contrast. The high kinetic stability of polymeric Gd-DOTA conjugate at physiological and acidic pH values (to simulate lysosomal conditions) is in agreement with the stability values for small molecular weight Gd-DOTA.

Results of cytotoxicity test showed lower toxicity ($p<0.019$) for polymeric conjugates at 72 h suggesting that gradual accumulation of polymeric contrast agent does not cause toxicity on breast cancer cell line. At 1000 μM equivalent of Gd, polymer-Gd conjugate showed significantly higher toxicity compared to Magnevist at 48 h ($p<0.025$) and 72 h ($p<0.010$) suggesting time dependent endocytosis of the polymeric complex at

high concentrations might be toxic. The same experiment was performed on NIH/3T3 cell line and showed lower trend of cytotoxicity on a healthy fibroblast cell line compared to a cancerous cell line at each concentration, probably due to higher uptake rate of the cancer cell line. The toxicity of polymeric drug conjugates with and without Gd on MDA-MB-435 (Figure 3) suggests that there is no interference between Gd and Dox effect.

Conclusion

In summary, HPMA copolymer – (DOTA-Gd) – Dox conjugates were synthesized and characterized. The conjugates were stable and showed higher relaxivity values than commercially available Gd-DOTA contrast agent (Dotarem®). The polymeric conjugate with Dox exhibited 1.6 times higher relaxivity than the polymeric conjugate without Dox. High relaxivity and stability of these conjugates coupled with low toxicity show the potential of these systems for monitoring the *in vivo* fate of HPMA copolymer-drug conjugates and further correlation of localization with efficacy in cancer treatment.

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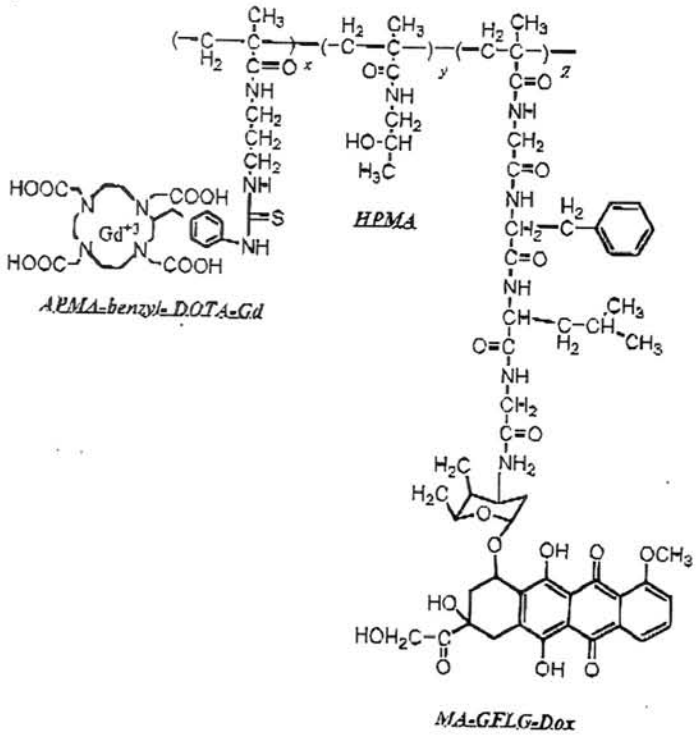

Figure 1. General structure of HPMA-copolymer-(DOTA-Gd)-Dox conjugates. (HPMA: *N*-(2-hydroxypropyl)methacrylamide; APMA-benzyl-DOTA: aminopropylmethacrylamide-benzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10-tetraacetic acid; Gd: gadolinium; MA-GFLG-Dox: Methacryloylglycylphenylalanylleucylglycyl-doxorubicin).

Sample[a]	Feed comonomer composition (mol%)	Polymer characteristics (mmol/g polymer)	Mw[b] (g/mol)	n[c]	Relaxivity ($s^{-1}mM^{-1}Gd$)

	HPMA	APMA-DOTA	MA-GFLG-Dox	Dox content	Gd content			
P-(DOTA-Gd)	90	10	0	-	0.41±0.014	35000	1.6	19.6
P-(DOTA-Gd)-Dox	85	10	5	0.26±0.05	0.19±0.021	34000	1.4	32.5

Table 1. Physicochemical characteristics of HPMA copolymer - contrast agent conjugates.

[a] For structures of polymer-contrast agent conjugates see Figure 1.

[b] Weight average molecular weight of polymer precursor.

[c] Polydispersity index.

Table 2. Stability of HPMA copolymer-(DOTA-Gd) complex as a function of pH.

Days	pH=3	pH=5	pH=7
	% Gd bound*		
1	95.9	97.5	99.1
3	89.9	95.9	98.9
5	85.8	95.9	98.5

* The data represent the means of duplicate points.

Table 3. Stability of HPMA copolymer-(DOTA-Gd) complex in the presence of EDTA.

No.	EDTA:Gd	Stability (% Gd bound)*
1	1:1	100
2	5:1	88.2
3	25:1	84.2
4	125:1	57.9

* The data represent the means of duplicate points.

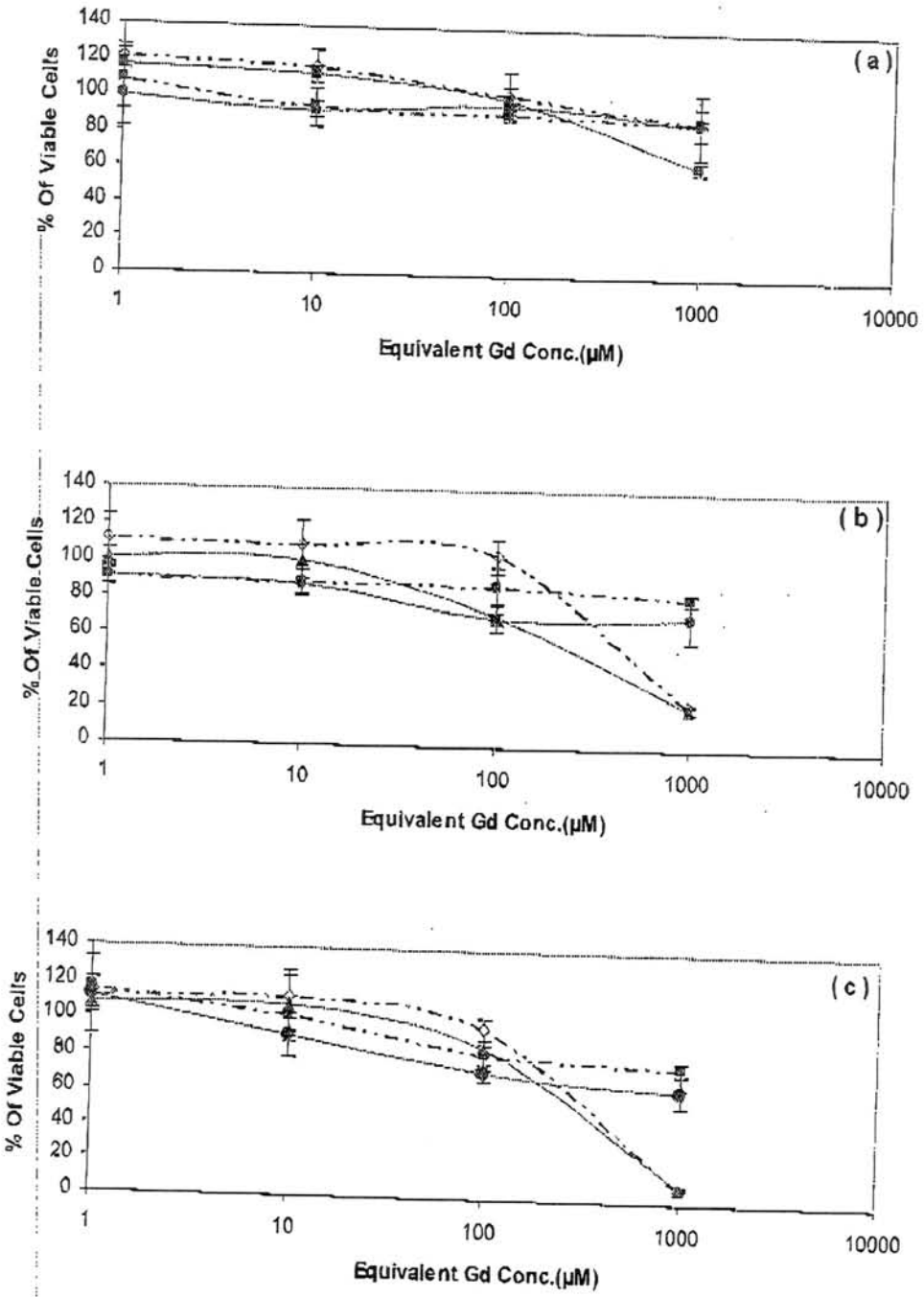

Figure 2. Comparison of cytotoxicity of varying concentrations of HPMA copolymer - Gd chelate using MTT assay on MDA-MB-435 and NIH/3T3 cell lines after : a) 24h; b) 48h; and c) 72h. P-Gd [NIH/3T3] (◊); Magnevist [NIH/3T3] (▪); P-Gd [MDA-MB-435] (▲); Magnevist [MDA-MB-435] (●);Data represent the means of triplicate ± standard error. For structures and characteristics of the samples see Figure 1 and Table 1

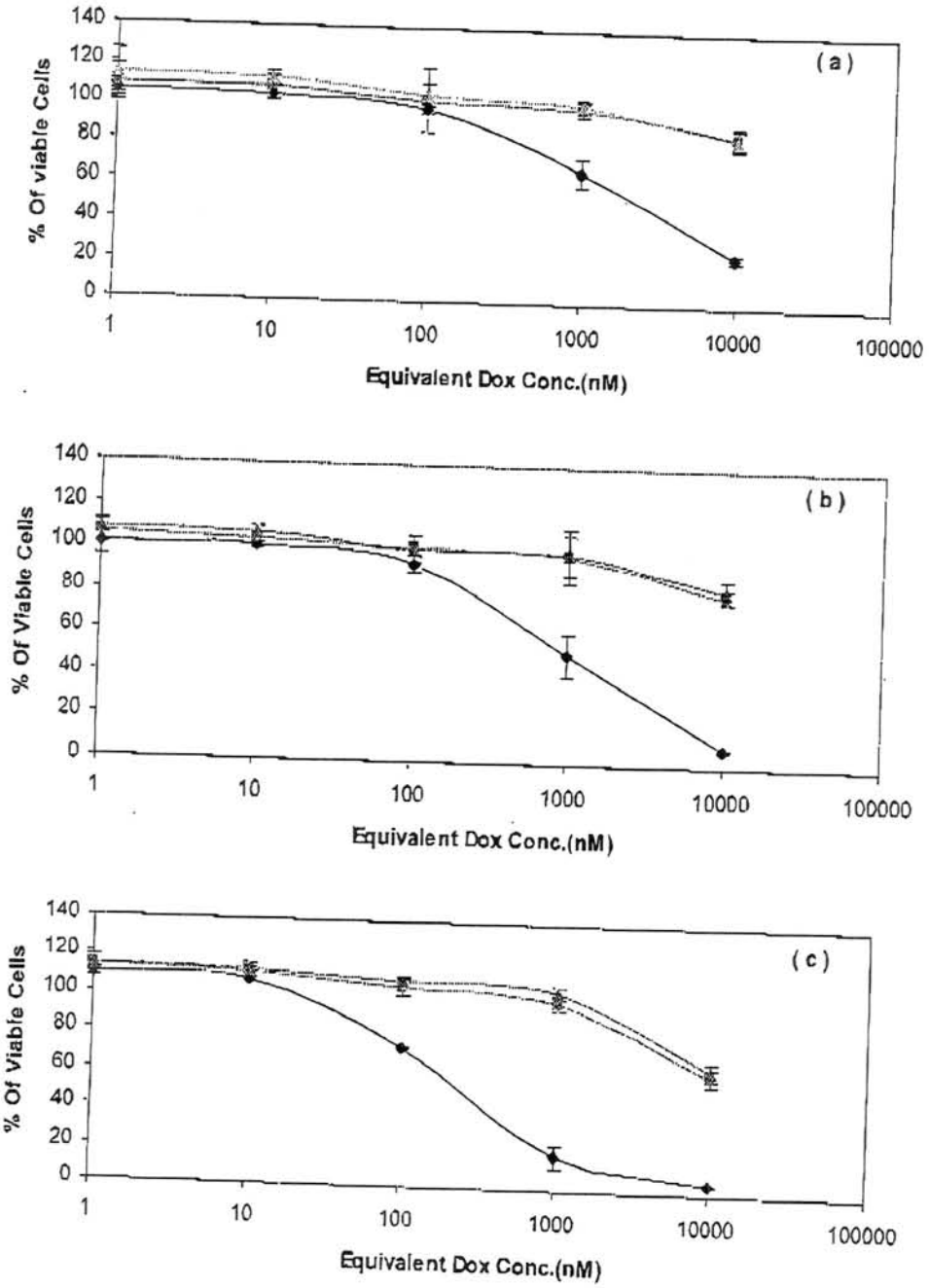

Figure 3. Effect of Gd on cytotoxicity of HPMA copolymer-Dox conjugates at 37°C after: a) 24h; b) 48h; and c) 72h. Dox (♦); P-Dox-DOTA (■); P-Dox-DOTA-Gd (▲) using MTT assay. Data represent the means of triplicate ± standard error. For structures and characteristics of the samples see Figure 1 and Table 1.

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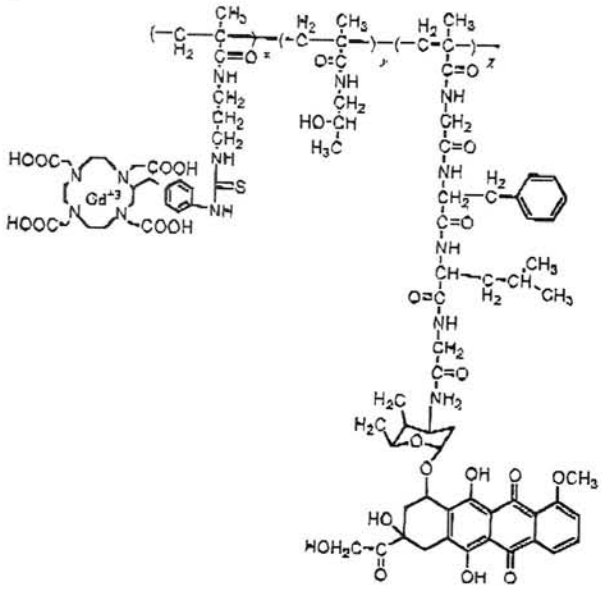
Communication: *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-gadolinium (Gd) – doxorubicin (Dox) conjugates as multifunctional drug delivery systems were synthesized, characterized and evaluated *in vitro*. Results showed high relaxivity and stability as well as low toxicity for these polymeric conjugates. These results demonstrate the potential of the copolymers for monitoring the fate of drug delivery systems by magnetic resonance imaging.



HPMA Copolymer-Doxorubicin-Gadolinium Conjugate.

Appendix 1

HPMA Copolymer- Doxorubicin- Gadolinium conjugates: Synthesis, Characterization and In Vitro Evaluation

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